Haemoglobin adducts of aromatic amines: aniline, *o*-, *m*- and *p*-toluidine, *o*-anisidine, *p*-chloroaniline, α- and β-naphthylamine, 4-aminodiphenyl, benzidine, 4,4′-diaminodiphenylmethane, 3,3′-dichlorobenzidine

Application	Determination of the adducts released from haemoglobin
Analytical principle	Gas chromatography/mass selective detection with negative chemical ionisation
Completed in	May 2000

Summary

Determination of the haemoglobin adducts of various aromatic amines can be achieved using this method. Excretion of the above-mentioned aromatic amines at levels of relevance to occupational medicine and those levels due to environmental exposure can be detected.

For this purpose erythrocytes are separated from EDTA-stabilised whole blood. The haemoglobin contained in the erythrocytes is isolated, and the aromatic amines are then cleaved from their conjugates with cysteine by means of a mild alkaline hydrolysis. The free amines are enriched and purified on an RP18 cartridge. After elution, the analytes are converted to their derivatives using heptafluorobutyric anhydride. The derivatives are separated by means of capillary gas chromatography and measured by a mass selective detector and negative chemical ionisation (NCI).

Calibration graphs are plotted with calibration standards prepared using bovine haemoglobin for quantitative evaluation. The calibration standards are treated in the same manner as the haemoglobin samples to be investigated. The detection limit of the

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method (determined in a high-resolution sector field mass spectrometer) is lower than 1 ng of the aromatic amines per litre blood. The background level found in the general public lies in this concentration range.

Aniline

Within-series imprecision:	Standard deviation (rel.)	$s_w = 4.5\%$
	Prognostic range	$u = 10.1\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 103\%$
Detection limit:	1 ng per litre blood	

o-Toluidine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 3.6\%$
	Prognostic range	$u = 8.1\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 122\%$
Detection limit:	1 ng per litre blood	

m-Toluidine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 6.2\%$
	Prognostic range	$u = 13.7\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 120\%$
Detection limit:	1 ng per litre blood	

p-Toluidine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 6.9\%$
	Prognostic range	$u = 15.3\%$

PM3006730685

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at a concentration of 10 ng per litre blood
and where $n = 10$ determinations

Accuracy: Recovery rate $r = 114\%$

Detection limit: 1 ng per litre blood

o-Anisidine

Within-series imprecision: Standard deviation (rel.) $s_w = 5.9\%$
Prognostic range $u = 13.1\%$
at a concentration of 10 ng per litre blood
and where $n = 10$ determinations

Accuracy: Recovery rate $r = 106\%$

Detection limit: 1 ng per litre blood

p-Chloroaniline

Within-series imprecision: Standard deviation (rel.) $s_w = 4.7\%$
Prognostic range $u = 10.5\%$
at a concentration of 10 ng per litre blood
and where $n = 10$ determinations

Accuracy: Recovery rate $r = 123\%$

Detection limit: 1 ng per litre blood

α-Naphthylamine

Within-series imprecision: Standard deviation (rel.) $s_w = 12.4\%$
Prognostic range $u = 27.7\%$
at a concentration of 10 ng per litre blood
and where $n = 10$ determinations

Accuracy: Recovery rate $r = 61\%$

Detection limit: 1 ng per litre blood

PM3006730686

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β-Naphthylamine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 12.2\%$
	Prognostic range	$u = 27.2\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 80\%$
Detection limit:	1 ng per litre blood	

4-Aminodiphenyl

Within-series imprecision:	Standard deviation (rel.)	$s_w = 3.8\%$
	Prognostic range	$u = 8.5\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 89\%$
Detection limit:	1 ng per litre blood	

Benzidine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 21.8\%$
	Prognostic range	$u = 48.7\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 72\%$
Detection limit:	1 ng per litre blood	

4,4′-Diaminodiphenylmethane

Within-series imprecision:	Standard deviation (rel.)	$s_w = 7.0\%$
	Prognostic range	$u = 15.7\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 62\%$
Detection limit:	1 ng per litre blood	

PM3006730687

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3,3′-Dichlorobenzidine

Within-series imprecision:	Standard deviation (rel.)	$s_w = 10.0\%$
	Prognostic range	$u = 45.1\%$
	at a concentration of 10 ng per litre blood and where $n = 10$ determinations	
Accuracy:	Recovery rate	$r = 111\%$
Detection limit:	1 ng per litre blood	

Aromatic amines

Most of the aromatic amines which can be determined by this method have carcinogenic properties. The majority of these aromatic amines can be adsorbed through the skin. Therefore, if the people who handle these substances come into direct skin contact with them, biomonitoring must be carried out as part of the preventive measures foreseen by occupational medicine (Technical Directive 150 of the German Regulations on Hazardous Substances – GefStoffV). As the general properties and the fundamentals relating to the toxicology of aromatic amines have already been discussed in this series [1–3], only current information on the MAK and BAT value lists of the Deutsche Forschungsgemeinschaft (Table 1) and some fundamental data on haemoglobin adducts will be given here. Further information can be obtained from the publications listed from [5–22] in the References section.

Table 1. Properties and exposure limits for aromatic amines, H=absorbed through the skin.

Substance	Absorbed through the skin	Category for classification of carcinogenic substances (DFG, [4])	Exposure limit
Aniline	H	3	MAK: 2 mL/m^3 BAT: 100 µg/L from Hb
o-Toluidine	H	2	TRK: 0.5 mg/m^3
m-Toluidine	Not classified in the MAK and BAT value list [4]		
p-Toluidine	H	3	TRK: 1 mg/m^3
o-Anisidine	H	2	TRK: 0.5 mg/m^3
p-Chloroaniline	H	2	TRK: 0.2 mg/m^3
α-Naphthylamine	Not classified in the MAK and BAT value list [4]		
β-Naphthylamine	H	1	cf. MAK list IX 2
4-Aminodiphenyl	–	1	cf. MAK list IX 2
Benzidine	H	1	cf. MAK list IX 2
4,4′-Diaminodiphenylmethane	H	2	TRK: 0.1 mg/m^3
3,3′-Dichlorobenzidine	H	2	TRK: 0.03 mg/m^3

PM3006730688

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Haemoglobin adducts

Aromatic amines activated to nitroso derivatives by the cytochrome P450 monooxigenase enzyme system can react with the terminal thiol functions of cysteine in haemoglobin to form (Hb) adducts in the form of sulphinic acid amides [14].
As the concentrations of aromatic amines are generally very low *in vivo*, pseudo first-order reaction kinetics are to be expected for the formation of adducts (DNA, Hb, etc.). Comparison of the adduct formation on DNA and haemoglobin is admissible, as the ratio of the product formation rates (DNA:Hb) and the ratio of the reaction constants (DNA:Hb) are the same under these conditions. Haemoglobin adducts can therefore be regarded as surrogates for DNA adducts. However, the appropriate Hb adduct must fulfil further conditions in this case [15, 16]:

1. The adduct must be stable under biological conditions.
2. The lifetime of the protein *in vivo* should not be influenced by the formation of the adduct.

Lars Ehrenberg and his co-workers were the first to recognise the potential for using protein adducts as a surrogate for DNA adducts [17]. The members of this working group were able to derive a mathematical relationship between continuous exposure to an alkylating agent and the "steady-state" adduct level by means of animal studies.
In man the lifetime of the erythrocytes and haemoglobin is approx. 120 days. The age distribution is homogeneous.
Therefore if a chemically and biologically stable Hb adduct is formed and exposure to the adduct-forming agent remains constant, then the amount of the adduct is proportional to the age of the haemoglobin. That means that the adduct concentration of Hb molecules which are 120 days old is 120 times higher than that of Hb molecules which are only one day old. As the number of 120-day-old erythrocytes is exactly the same as the number of one-day-old erythrocytes (homogeneous age distribution), it follows that the mean adduct concentration in man is approx. 60 times the amount which would be formed by a single-day dose.
Even when the daily dose is not constant or when the exposure is interrupted, the adduct amount is equivalent to 60 times the mean daily dose during the previous 120 days. Thus the mean exposure over 4 months can be estimated by monitoring the biochemical effect on Hb.
It should be noted, however, that not all carcinogenic aromatic amines form adducts which are so stable that their lifetime *in vivo is* equivalent to that of unchanged erythrocytes [18].

Authors: *J. Lewalter, W. Gries*
Examiners: *J. Angerer, G. Sabbioni*

PM3006730689

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Contents

1 General principles
2 Equipment, chemicals and solutions
2.1 Equipment
2.2 Chemicals
2.3 Solutions and conditioning of the extraction material
2.4 Calibration standards
3 Specimen collection, storage, sample preparation and sample processing
3.1 Specimen collection
3.2 Storage
3.3 Sample preparation
3.4 Sample processing
4 Operational parameters
4.1 Operational parameters for gas chromatography
4.2 MS operational conditions
5 Analytical determination

PM3006730690

Source: https://www.industrydocuments.ucsf.edu/docs/pyvj0001

6 Calibration
7 Calculation of the analytical result
8 Standardisation and quality control
9 Reliability of the method
9.1 Precision
9.2 Accuracy
9.3 Detection limits
9.4 Sources of error
10 Discussion of the method
Addendum: Isolation of haemoglobin without drying step

1 General principles

After isolation of the haemoglobin (Hb) from the separated erythrocytes, the aromatic amines are then cleaved from their conjugates with cysteine by means of a mild alkaline hydrolysis. The released amines are enriched and purified on an RP18 cartridge. After elution, the analytes are converted to their derivatives using heptafluorobutyric anhydride. The derivatives are separated by capillary gas chromatography and measured by means of a mass selective detector and negative chemical ionisation (NCI). Calibration graphs are plotted with calibration standards prepared using bovine haemoglobin for quantitative evaluation. The calibration standards are treated in the same manner as the haemoglobin samples to be investigated. The detection limit of the method (determined in a high-resolution sector field mass spectrometer) is lower than 1 ng of the aromatic amines per litre blood. The background level found in the general public lies in this concentration range.

2 Equipment, chemicals and solutions

2.1 Equipment

Gas chromatograph, mass selective detector with the possibility of negative chemical ionisation and an autosampler

Fused silica capillary column 25 m×0.2 m×0.33 µm, 95% dimethyl-5% diphenylpolysiloxane (e.g. DB5 MS from J&W Scientific)

Disposable syringes containing an anticoagulant (e.g. potassium EDTA Monovettes®, from Sarstedt)

Haematocrit centrifuge

Microcapillary, 20 µL, calibrated
Laboratory centrifuge

PM3006730691

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Water bath, thermostatically controlled at 25 °C

Shaker (e.g. Vortex from Cenco, Netherlands)

Water jet pump or vacuum pump

Vacuum desiccator

Vacuum centrifuge

Dispensers with settings from 0 to 25 mL (e.g. from Brandt)

Magnetic stirrer

Volumetric flasks, 10 mL and 100 mL

Glass tubes (50 mL diameter: 2.5 cm, height: 15 cm)

50 mL conical test-tube (e.g. Falcon® 2070 Blue Max)

Teflon centrifuge tubes 10 mL (e.g. Nalgene® from Oak Ridge FEP)

200 µL conical microvials (e.g. Chromacol® 02-CTV6)

Transfer pipettes, 3.5 mL and 1 mL (e.g. from Sarstedt)

Na_2SO_4 filter:
The filter must be made in the laboratory. For this purpose a plug of cotton wool is inserted into a plastic Pasteur pipette. The pipette is then filled with approx. 1 g anhydrous sodium sulphate.

Microlitre pipettes, adjustable between 1 and 10 µL, 10 and 100 µL, and 100 and 1000 µL (e.g. from Eppendorf)

Suction flask and suction filter

Vacuum station for solid phase extraction (e.g. from Baker)

2.2 Chemicals

Ethanol, p.a. (e.g. from Merck)

Diethyl ether Uvasol (with EtOH as a stabiliser) (e.g. from Merck)

Acetonitrile for chromatography (e.g. from Merck)

PM3006730692

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Surgical cotton wool

Aniline (e.g. from Sigma-Aldrich)

o-, *m*-, *p*-Toluidine (e.g. from Sigma-Aldrich)

o-Anisidine (e.g. from Sigma-Aldrich)

p-Chloroaniline (e.g. from Sigma-Aldrich)

α-, β-Naphthylamine (e.g. from Sigma-Aldrich)

4-Aminodiphenyl (e.g. from Sigma-Aldrich)

4,4′-Diaminodiphenylmethane (e.g. from Dr. Ehrenstorfer)

Benzidine (e.g. from Sigma-Aldrich)

3,3-Dichlorobenzidine (e.g. from Sigma-Aldrich)

4-Aminodiphenyl-d_9 (e.g. from CDN Isotopes)

o-Toluidine-d_9 (e.g. from CDN Isotopes)

3,3′-Diaminodiphenylmethane (e.g. from Bayer AG)

3,3′-Dichlorobenzidine-d_6 (e.g. from CDN Isotopes)

Milli Q water

RP 18 separation columns (end-capped, 500 mg) (e.g. from Baker Art. No. 7020-06)

Bovine haemoglobin (e.g. from Fluka 51290)

Heptafluorobutyric anhydride (HFBA) (e.g. from Fluka)

Acetonitrile SupraSolv (e.g. from Merck)

Toluene SupraSolv (e.g. from Merck)

Sodium hydroxide, ultrapure (e.g. from Merck)

Sodium dihydrogen phosphate (e.g. from Merck)

Sodium sulphate, anhydrous for trace analysis (e.g. from Merck)

PM3006730693

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Chloroform SupraSolv (from Merck)

Physiological saline solution

2.3 Solutions and conditioning of the extraction material

0.1 N Sodium hydroxide:
4.0 g NaOH are weighed in a 1000 mL volumetric flask and dissolved in about 300 mL ultrapure water. After the solution is swirled, the volumetric flask is filled to its nominal volume with ultrapure water and mixed well.

0.2 M Sodium dihydrogen phosphate buffer (pH 7.0):
24 g (0.2 M) NaH_2PO_4 are weighed in a 1000 mL volumetric flask and dissolved in approx. 300 mL ultrapure water. After the contents have been swirled, the volumetric flask is filled to its nominal volume with ultrapure water. The pH value is subsequently adjusted to pH 7.0 using conc NaOH.

Conditioning the RP18 column:
a) The column is first rinsed with 6 mL methanol,
b) then it is rinsed with 6 mL water and finally
c) the column is rinsed with 6 mL 0.05 M NaOH.

2.4 Calibration standards

Starting solution:
Approx. 10 mg of the aromatic amines are each weighed exactly in separate 10 mL volumetric flasks and dissolved in acetonitrile. Then the flasks are filled to their nominal values with acetonitrile (exception: 3,3'-dichlorobenzidine, solvent: methanol). These solutions can be stored in the refrigerator for at least 1 month. The concentration of the aromatic amines in these solutions is 1 g/L.

Stock solution:
100 µL of each starting solution are pipetted into a 100 mL volumetric flask and the flask is filled to its nominal value with acetonitrile. The concentration of the aromatic amines in this solution is 1 mg/L.

Spiked solution A:
1000 µL of the starting solution are pipetted into a 10 mL volumetric flask and the flask is filled to its nominal value with toluene. The concentration of the aromatic amines in this solution is 100 µg/L.

Spiked solution B:
100 µL of the stock solution are pipetted into a 10 mL volumetric flask and the flask is filled to its nominal value with toluene. The concentration of the aromatic amines in this solution is 10 µg/L.

PM3006730694

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Spiked solution C:
100 µL of spiked solution A are pipetted into a 10 mL volumetric flask and the flask is filled to its nominal value with toluene. The concentration of the aromatic amines in this solution is 1 µg/L.

The calibration standards are prepared by spiking bovine haemoglobin (200 mg Hb dissolved in 5 mL 0.1 N NaOH) in a sample vial with a screw cap. The pipetting scheme for the preparation is shown in Table 2. The error resulting from dilution is negligible. These calibration standards must be freshly prepared before each analytical series.

Table 2. Pipetting scheme.

Internal standard [µL]	Volume of the spiked solutions [µL]			Concentration of the calibration standards in blood [ng/L]	Concentration of the calibration standards pg/ 1.25 mL blood or 200 mg Hb
	C	B	A		
10	1.25	–	–	1	1.25
10	2.5	–	–	2	2.5
10	6.25	–	–	5	6.25
10	12.5	–	–	10	12.5
10	–	2.5	–	20	25
10	–	6.25	–	50	62.5
10	–	12.5	–	100	125
10	–	–	2.5	200	250
10	–	–	6.25	500	625
10	–	–	12.5	1000	1250

Internal standards

Starting solution for internal standards:
Several internal standards are used: *o*-toluidine-d_9, 4-aminodiphenyl-d_9, 3,3′-diaminodiphenylmethane und 3,3′-dichlorobenzidine-d_6.
Approx. 10 mg of the internal standards are each weighed exactly in separate 10 mL volumetric flasks and dissolved in acetonitrile. Then the flasks are filled to their nominal values with acetonitrile (exception: 3,3′-dichlorobenzidine-d_6, solvent: methanol). These solutions can be stored in the refrigerator for at least 1 month. The concentration of the internal standards in these solutions is 1 g/L in each case.

Internal standard solution:
10 µL of each starting solution of the internal standards are pipetted into a 100 mL volumetric flask and the flask is filled to its nominal value with toluene. The concentration of this solution is 100 µg/L.

PM3006730695

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3 Specimen collection, storage, sample preparation and sample processing

3.1 Specimen collection

After collection in EDTA Monovettes the blood sample should be processed as soon as possible and must be processed at the latest after 3 days storage at 4–6 °C. A blood sample of at least 6 mL should be taken.

3.2 Storage

Blood samples:
The blood samples can be stored for 24 hours at room temperature and for up to a maximum of 3 days at 4–6 °C. The blood samples may not be deep-frozen before the erythrocytes have been isolated.

Erythrocyte samples:
It is advisable to isolate the erythrocytes immediately after the blood sample is collected. 7.5 mL distilled water are added to the isolated erythrocytes (approx. 2.5 mL), this mixture is homogenised and it can then be stored for an indefinite period of time at approx. –20 °C.

Haemoglobin samples:
Dried haemoglobin can be stored for approx. 1–2 weeks at 4–6 °C in the refrigerator or deep-frozen at –20 °C for an indefinite period.

3.3 Sample preparation

Isolation of the erythrocytes:
Blood samples of 5 mL are centrifuged for 5 min at 1200 g. The plasma is drawn off using a pipette.
2.5 mL of the 0.9% (154 mmol) sodium chloride solution are added to the erythrocyte sediment and they are mixed thoroughly. Centrifugation for 5 min at 1200 g is repeated. The supernatant is drawn off using a pipette and discarded. This washing step is repeated until the supernatant is colourless (at least twice). Approximately 2.5 mL erythrocytes should be isolated from 5 mL blood.
Then 7.5 mL distilled water are added to the isolated and purified erythrocytes and they are deep-frozen.

Isolation of haemoglobin:
The frozen lysate sample is thawed and then homogenised by shaking.
The sample is centrifuged for about 10 min at 2000 g to remove the cell debris. 5 mL of the supernatant are drawn off, devided between two glass tubes (50 mL, diameter: 2.5 cm, height: 15 cm), and warmed to 25 °C in a water bath. While the tube is being

PM3006730696

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shaken (Vortex), 12.5 mL ethanol are rapidly added to the Hb (each tube) by means of a dispenser. The shaking procedure may not be interrupted, as otherwise the haemoglobin is not completely precipitated.

After addition of the ethanol, shaking must be continued (Vortex), whereby the colour darkens from pale red to brownish-red, for about 1 min until no further discoloration can be observed. The precipitated haemoglobin should now be present as fine particles. The haemoglobin is allowed to settle, whereby the upper ethanol/H_2O phase must be colourless.

Approximately 4/5 of the supernatant are decanted off and discarded. A residue of the EtOH/H_2O phase must be present, as otherwise the haemoglobin cannot be shaken up. The content of the tube is mixed by shaking vigorously, and the mixture is completely transferred into 125 mL H_2O. If processing has been correctly carried out, the haemoglobin will settle slowly at this point (approx. 1 hour). The H_2O phase should once again be colourless. Then the water is sucked off the haemoglobin over a black band vacuum filter. It is important to ensure that the filtered residue is not sucked dry. It should be washed immediately with 125 mL H_2O to remove any glutathione residues. The haemoglobin should be re-suspended during this procedure.

The supernatant water is largely sucked off, but it is important at this stage to ensure that the haemoglobin always remains moistened with water. Subsequently the water is removed in steps by suspending the haemoglobin in 25 mL ethanol/water (2:1; v:v) and then 25 mL ethanol/ether (1:3; v:v) and sucking the liquid off in each case. Finally the haemoglobin is re-suspended in 40 mL ether (p.a., with EtOH as a stabiliser) without applying a vacuum. The haemoglobin is allowed to settle and the liquid to drain away without applying a vacuum.

After about 5 min waiting time the haemoglobin is sucked dry with air under a vacuum and transferred to conical 50 mL test-tubes (e.g. Falcon® 2070 Blue Max) and dried in a vacuum desiccator.

3.4 Sample processing

Approx. 200 mg of the dried haemoglobin are weighed exactly in separate test-tubes with screw caps, then 5 mL 0.1 M NaOH and 10 µL of the solution of the internal standards are added. The sample is mechanically shaken for one hour to ensure hydrolysis is carried out. Then the sample is diluted to approx. 10 mL with water. Any insoluble components which may be present are separated by centrifugation (10 minutes at 2000 g). The supernatant is introduced onto the RP18 column conditioned as described in Section 2.3, and is allowed to trickle slowly through at atmospheric pressure. If necessary a slight vacuum can be applied. After the sample has trickled through, the column is washed with 2 mL 0.1 N sodium hydroxide and subsequently centrifuged for about 20 min at a minimum of 2000 g to remove any residues of the sodium hydroxide solution.

The aromatic amines are now eluted from the column using 3 mL chloroform, whereby the eluate passes through a Na_2SO_4 filter into a Teflon tube. The Na_2SO_4 filter is washed with approx. 1 mL chloroform and the filtrate is also transferred to the Teflon

PM3006730697

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tube. Derivatisation is accomplished by adding 10 µL HFBA to the solution and shaking the tube for 10 min at room temperature on the Vortex shaker. The derivatisation reaction is subsequently stopped by the addition of 50 µL methanol. Then 250 µL toluene are added as a keeper and the sample is evaporated to approx. 150 µL in a vacuum centrifuge. The sample is than washed with 0.5 mL phosphate buffer. After separation of the layers the toluene phase containing the analyte is then transferred to a GC microvial, evaporated to approx. 50 µL in a stream of nitrogen, and finally analysed by means of GC-MS.

4 Operational parameters

4.1 Operational parameters for gas chromatography

Capillary column:	Material:	Fused silica
	Stationary phase:	DB5-MS
	Length:	25 m
	Inner diameter:	0.2 mm
	Film thickness:	0.33 µm
Detector:	Mass selective detector (MSD)	
Temperatures:	Column:	1 min at 80 °C; then increase by 12 °C/min until 210 °C; than increase by 15 °C/min until 320 °C; 10 min at the final temperature
	Injector:	280 °C
	Transfer line:	250 °C
Carrier gas:	Helium with a column pre-pressure of 80 kPa	
Split:	Splitless, split on after 1 min	
Septum purging:	3 mL/minute	
Sample volume:	1 µL	

4.2 MS operational conditions

Ionisation mode:	Negative chemical ionisation with methane as the reactant gas at 70 eV
Resolution:	5000
Accelerating voltage:	8000 V
Multiplier voltage:	330 V
Trap current:	300 µA
Source temperature:	190 °C

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5 Analytical determination

The given operational parameters for the instruments are adjusted and 1 µL of the analytical sample is injected into the gas chromatograph in each case. Figure 1 shows the GC-MS chromatogram of a sample of bovine haemoglobin spiked with 100 ng/L of the aromatic amines.

If the measured values are higher than the range covered by the calibration graphs, the Hb samples are diluted using 0.1 N NaOH in the ratio of 1:10 then processed and analysed anew. A quality control sample is analysed with each analytical series. Reagent blank values are to be expected. Therefore a blank sample (5 mL 0.1 N NaOH) must be included in each analytical series. It must be processed and analysed as described in these instructions.

Quantification of the individual aromatic amines is based on the masses shown in Table 3.

Table 3. Retention times and the masses used for quantification.

Compound	Retention time [min]	Mass [g/mol]
Aniline-HFBA	5.36	269.0275
o-Toluidine-HFBA	6.15	283.0432
m-Toluidine-HFBA	6.39	283.0432
p-Toluidine-HFBA	6.48	283.0432
o-Toluidine d_9-HFBA (IS)	6.12	290.0871
o-Anisidine-HFBA	7.28	299.0381
p-Chloroaniline-HFBA	7.45	302.9886
α-Naphthylamine-HFBA	10.45	319.0432
β-Naphthylamine-HFBA	11.24	319.0432
4-Aminodiphenyl-HFBA	13.14	345.0588
4-Aminodiphenyl-d_9-HFBA (IS)	13.12	354.1153
Benzidine-$(HFBA)_2$	16.28	556.0456
3,3'-Diaminodiphenylmethane-$(HFBA)_2$ (IS)	15.39	570.0613
4,4'-Diaminodiphenylmethane-$(HFBA)_2$	16.48	570.0613
3,3'-Dichlorobenzidine-$(HFBA)_2$	17.04	625.9647
3,3'-Dichlorobenzidine-d_6-$(HFBA)_2$ (IS)	17.03	630.0054

IS = internal standard

6 Calibration

The haemoglobin calibration standards are processed and analysed in the same manner as the assay samples. Calibration graphs are obtained by plotting the quotients of the peak areas for each aromatic amine and that of the appropriate internal standard as a function of the concentrations used.

o-Toluidine-d_9 is used as an internal standard to quantify aniline, *o*-, *m*- and *p*-toluidine as well as for *o*-anisidine and *p*-chloroaniline. Quantification of *α*- and *β*-naph-

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tylamine as well as 4-aminodiphenyl and benzidine is based on 4-aminodiphenyl-d_9. 3,3′-diaminodiphenylmethane is used for the quantification of 4,4′-diaminodiphenylmethane and 3,3′-dichlorobenzidine-d_6 for the determination of 3,3′-dichlorobenzidine. The calibration functions of the aromatic amines are each linear in the concentration range between 0.001 and 1.0 µg/L. This was checked using aqueous standard solutions.

7 Calculation of the analytical result

The resulting peak area is divided by the peak area of the relevant inner standard. The quotients thus obtained are used to read off the corresponding concentrations of the analytes in µg/L or ng/L blood from the appropriate calibration graph. If the haemoglobin used to prepare the calibration standards exhibits background levels, the resulting calibration graph must be shifted in parallel so that it passes through the zero point of the coordinates. Any reagent blank values must be subtracted from the analytical results for the real samples.

8 Standardisation and quality control

A quality control standard to check the precision of the method must be included in each analytical series as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) [23, 24] and in the special preliminary remarks to this series.
As no reference material is commercially available, it must be prepared in the laboratory. For this purpose, bovine or human haemoglobin (Hb dissolved in 0.1 N NaOH, concentration 40 g per litre 0.1 N NaOH) is spiked with a defined quantity of the aromatic amines. Aliquots of this solution can be stored in the deep-freezer for up to a year and used for quality control. The concentration of this control material should lie in the middle of the most frequently occurring concentration range. The theoretical value and the tolerance range for this quality control material is determined in the course of a pre-analytical period (one analysis of the control material on 20 different days) [25, 26].

9 Reliability of the method

9.1 Precision

The precision of the method was checked by repeated determination ($n = 10$) of the peak areas of the analytes relative to the peak areas of the appropriate internal standard at two different concentrations. The relative standard deviation was between 2.2% for 4-aminodiphenyl and 37.5% for α-naphtylamine (Table 4).

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Table 4. Precision in the series for 10 and 100 ng/L (spiked bovine haemoglobin ($n=10$)).

Substance	Concentration [ng/L]	Standard deviation (rel.) [%]	Prognostic range [%]
Aniline	10	4.5	10.1
	100	6.5	14.4
o-Toluidine	10	3.6	8.1
	100	11.9	26.6
m-Toluidine	10	6.2	13.7
	100	9.5	21.1
p-Toluidine	10	6.9	15.3
	100	12.9	28.8
o-Anisidine	10	5.9	13.1
	100	8.0	17.8
p-Chloroaniline	10	4.7	10.5
	100	9.6	21.4
α-Naphthylamine	10	12.4	27.7
	100	37.5	83.6
β-Naphthylamine	10	12.2	27.2
	100	13.3	29.6
4-Aminodiphenyl	10	3.8	8.5
	100	2.2	4.9
Benzidine	10	21.8	48.7
	100	17.3	38.6
4,4′-Diaminodiphenylmethane	10	7.0	15.7
	100	5.9	13.1
3,3′-Dichlorobenzidine	10	10.0	45.1
	100	3.0	6.6

9.2 Accuracy

Investigations were carried out to ascertain the losses of aromatic amines which occurred during processing. For this purpose the results for the spiked and processed samples were compared with those for unprocessed standards prepared in an organic solvent. The losses due to processing are documented Table 5.

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Table 5. Losses due to sample processing.

Substance	Concentration [ng/L]	Losses due to processing [%]
Aniline	100	–
o-Toluidine	100	6
m-Toluidine	100	15
p-Toluidine	100	27
o-Anisidine	100	22
p-Chloroaniline	100	–
α-Naphthylamine	100	69
β-Naphthylamine	100	34
4-Aminodiphenyl	100	12
Benzidine	100	25
4,4′-Diaminodiphenylmethane	100	39
3,3′-Dichlorobenzidine	100	19

9.3 Detection limits

Based on a signal to background ratio of 1:5, the detection limits for the aromatic amines measured with this method were each approx. 1 ng/L. This theoretical detection limit of the method cannot be checked in practice for the ubiquitously occurring aromatic amines, as it lies below the background levels.

9.4 Sources of error

When isolating the haemoglobin, it is important to ensure that the ethanol/water phase is colourless. If it is still coloured, precipitation losses and difficulties in the further processing of the haemoglobin (clogging of the column) are to be expected.

The derivatisation reaction can only proceed to completion and is only reproducible in an anhydrous milieu. The chloroform eluate must therefore be carefully dried over sodium sulphate. A plastic Pasteur pipette filled with sodium sulphate has proved successful for this purpose. It can be directly connected to the end of the RP18 cartridge.

After isolation of the haemoglobin, no glass vessels should be used in the microtrace analysis of the aromatic amines in order to prevent adsorption at "active sites". In addition the extracts should not be evaporated to complete dryness to prevent losses of the analytes due to evaporation. A nitrogen evaporator should not be used instead of the recommended vacuum centrifuge (SpeedVac) for the first concentration step in the ultratrace range (< 10 ng/L), as considerable losses were observed by the author.

Quantification, especially of polyaromatic amines, in the trace range is highly dependent on the matrix.

Optimum quantification of the aromatic amines from haemoglobin adducts is achieved only with the use of several deuterated internal standards. Their behaviour during

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analysis closely resembles that of the target substances. These standards must be added to the sample before beginning sample preparation.
Measurements with the sector field MS can be impeded by masses overlapping with the signal of the calibration gas. In such cases the detection sensitivity drops, while the background noise increases. These effects were observed in the case of dichlorobenzidine. Such problems can be solved by pumping off the calibration gas completely.

10 Discussion of the method

The haemoglobin adducts of the aromatic amines formed as a result of occupational exposure can be reproducibly analysed using the method described here. After being released from their adducts, the various aromatic amines are adsorbed on RP18 cartridges and separated from the concomitant substances. In contrast to the conventional liquid-liquid extraction, this prevents interfering emulsions being formed when samples containing proteins are processed. Various methods for isolating haemoglobin from blood are given in the literature. Glutathione adducts which are present could also be precipitated under certain circumstances. In the case of short-term acute exposure (=glutathione adducts) this would lead to overestimation of the mean exposure (=haemoglobin adducts). The precipitation method given in these instructions reduces the presence of possible glutathione adducts. The haemoglobin precipitation method of Albrecht and Neumann [11, 12] is less laborious and more suitable for routine analyses, but the glutathione adducts are not as completely eliminated. When erythrocyte lysates are spiked with glutathione adducts of aromatic amines, the amount of glutathione adducts recovered is 5 times less after using this precipitation method than after using Neumann's precipitation method, i.e. 0.2% instead of 1% [13].
Strictly speaking, the haemoglobin should be isolated and dried before hydrolysis and 200 mg should be weighed and subjected to further processing. On average 200 mg (190–212 mg) haemoglobin are isolated from 1.25 mL EDTA-stabilised whole blood. The fluctuation range in the recovery of haemoglobin is only ±3% in this case. Thus intermediate isolation and weighing of the haemoglobin fraction can be dispensed with in practice, and haemoglobin separated directly from 1.25 mL EDTA blood without a drying step can be used. Instructions for the isolation without drying of haemoglobin are to be found in the addendum to this method.
All the quantitative analytical results were based on a starting volume of 5 mL blood and converted to give the value with respect to 1 litre blood.
The sensitivity of the method permits the detection of haemoglobin adducts in the general population who are exposed only to background levels, and it also permits biomonitoring of occupationally exposed subjects. This method not only enables analysis of the explicitly investigated aromatic amines but also a multitude of further aromatic amines down to the environmentally relevant range. The suitability of the method must be checked with respect to its reliability criteria in each individual case (see also Addendum).
The reliability criteria of the method described here can be designated as good. However, the imprecision is also greater than 20% in some cases (α-naphtylamine and benzidine). At concentrations of less than 50 to 100 ng/L higher errors must be expected,

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e.g. recovery rates of 150% are observed (mainly in the case of the mononuclear aromatic amines). With the exception of α-naphtylamine, the losses due to processing can be described as low. Matrix effects impede the quantification, especially of the polyaromatic aromatic amines, in the ultratrace range. This was improved by the use of additional specific deuterated internal standards.
The background levels of ubiquitously occurring aromatic amines, such as aniline, *o*-, *m*- and *p*-toluidine, *o*-anisidine, *p*-chloroaniline, etc. in the haemoglobin samples makes the evaluation of the inner exposure difficult in the case of investigations in occupational medicine.
On account of the high precision of the sector field MS instrument it is unnecessary to measure a second or further masses to ensure more accurate results.
Thus the method meets the requirements of preventive occupational and environmental medicine for monitoring the exposure of people who come into contact with diverse aromatic amines.
In the form presented here the method permits the analysis of a large series of samples. The detection sensitivity can be further enhanced by the use of on-column injection techniques.

Instruments used:
Autospec Ultima from Micromass; gas chromatograph HP 5890/II from Hewlett-Packard and CTC A200S autosampler.

Addendum

Isolation of haemoglobin without drying step

As described in Section 3.3, erythrocytes are isolated from 5 mL whole blood then 7.5 mL water is added and the mixture is frozen.
The frozen 10 mL lysate sample is thawed and then homogenised by shaking.
The sample is centrifuged for about 10 minutes at 2000 g to remove the cell debris. The supernatant is drawn off, divided between two glass tubes (50 mL, diameter: 2.5 cm, height: 15 cm), and heated to 25 °C in a water bath. While the tube is being shaken (Vortex), 12.5 mL ethanol are rapidly added to each of the two tubes by means of a dispenser. The shaking procedure may not be interrupted, as otherwise the haemoglobin is not completely precipitated.
After addition of the ethanol, shaking must be continued (Vortex), whereby the colour darkens from pale red to brownish-red, for about 1 min until no further discoloration can be observed. The precipitated haemoglobin should now be present as fine particles. The haemoglobin is allowed to settle, whereby the upper ethanol/H_2O phase must be colourless. Approximately 4/5 of the supernatant are decanted off and discarded. A residue of the ethanol/H_2O phase must be present, as otherwise the haemoglobin cannot be shaken up. The contents of both tubes are mixed together while being shaken vigorously, and the mixture is completely transferred into 125 mL H_2O.

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If processing has been correctly carried out, the haemoglobin will settle slowly at this point (approx. 1 hour). The H_2O phase should be colourless.
Then the water is sucked off the haemoglobin over a black band vacuum filter. It is important to ensure that the filtered residue is not sucked dry. It should be washed immediately with 125 mL H_2O to remove any glutathione residues. The haemoglobin should be re-suspended during this procedure.
The supernatant water is largely sucked off, but it is important at this stage to ensure that the haemoglobin always remains moistened with water. The filter residue is transferred to a test-tube with a screw cap, then 5 mL 0.1 M NaOH and 10 µL of the solution of the internal standards are added. The sample is mechanically shaken and further processing is carried out as described in Section 3.4.

Alternative methods

Aniline, *o*-, *m*- and *p*-toluidine, *p*-chloroaniline, *o*-anisidine, *α*-naphthylamine, *β*-naphthylamine, 4-aminodiphenyl, benzidine, 4,4′-methylendianiline and 3,3′-dichlorebenzidine are detected by the suggested method. 2,6-Toluenediamine cannot be satisfactorily determined, and it proved impossible to quantify 2,4-toluenediamine with this method. Moreover, the monoacetylated diamines, in particular N-acetylbenzidine and N-acetyl-4,4′-diaminophenylmethane, are not detected by the suggested method.
Therefore, three alternative possibilities for determination are described below. One of these methods is based on a solid phase extraction, the other two methods on liquid/liquid extraction.

Haemoglobin adducts of monoamines and diamines as well as of singly acetylated difunctional amines (liquid/solid)

200 mg dry and finely powdered haemoglobin are suspended in 5 mL water and 1 mL conc. sulphuric acid in a test-tube with a screw cap and subsequently incubated at 80 °C for 2 h to carry out hydrolysis. After the sample has cooled, 4 mL 32% NaOH are added slowly. The pH value must remain above 9.
After spiking with 10 µL of the solution of the internal standard, the samples are diluted with water to 10 mL. Any insoluble components which may be present are separated by centrifugation (10 minutes at 2000 g). The sample is introduced onto the RP18 column conditioned as described in Section 2.3. and is allowed to trickle slowly through at atmospheric pressure. If necessary a slight vacuum should be applied. After the sample has trickled through, the column is washed with 2 mL 0.1 N sodium hydroxide and subsequently centrifuged for about 20 min at a minimum of 2000 g to remove any sodium hydroxide residues.
The aromatic amines are now eluted from the column using 3 mL chloroform, whereby the eluate passes through a connected Na_2SO_4-filter into a Nalgene Teflon tube. The Na_2SO_4 filter is washed with approx. 1 mL chloroform and the filtrate is also transferred to the Nalgene Teflon tube. Derivatisation is accomplished by adding

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10 µL HFBA to the solution and shaking the tube for 10 minutes at room temperature on the Vortex shaker. The derivatisation reaction is subsequently stopped by the addition of 50 µL methanol. Then 250 µL toluene are added as a keeper, and the sample is evaporated to approx. 150 µL in a vacuum centrifuge. The sample is washed with 0.5 mL phosphate buffer and the buffer phase is drawn off and discarded. The toluene phase is transferred to a GC microvial, evaporated to approx. 50 µL in a stream of nitrogen and finally analysed by means of GC-MS under the conditions described in Section 4.

Two liquid-liquid extraction methods are also suitable as alternatives. They were developed by the Sabbioni working group and were submitted for assessment to the Deutsche Forschungsgemeinschaft's Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. The polyaromatic amines are analysed together with the diamines [19–21], but separately from the readily volatile aromatic amines [22] by means of the liquid-liquid extraction method.

Haemoglobin adducts of polyaromatic amines and diamines (liquid/liquid) [19]

0.1 M sodium hydroxide (4 mL), 24 µg 4-methylaniline and the recovery standards N'-acetyl-4,4'-diaminophenylmethane-d_4, N'-acetylbenzidine-d_4, 2-aminofluorene-d_9, 3-aminofluoranthene-d_8, benzidine-d_4, 3,3'-dichlorobenzidine-d_6, 3,3'-dimethylbenzidine-d_4, 4,4'-diaminophenylmethane-d_4, 2,4-toluenediamine-d_6, and 2,6-toluenediamine-d_6 are each added to haemoglobin (200 mg) and repeatedly shaken until the mixture is completely dissolved. After being stirred for one hour at room temperature, the aqueous phase is extracted with dichloromethane (6 mL) for 3 minutes. In order to eliminate any possible intermediate phases the samples are centrifuged, frozen using liquid nitrogen, and thawed. The organic phase is dried by passing it through a Pasteur pipette filled with sodium sulphate, and the sodium sulphate is subsequently washed with dichloromethane (1 mL). Heptafluorobutyric anhydride (HFBA) (2 µL) is added to the combined extracts. After 10 min at room temperature, the reaction is stopped with 20 µg 4-methylaniline in 50 µL methanol and the solution is evaporated to dryness in a water-jet vacuum in the SpeedVac at approx. 40 °C. The residue is transferred to sample vials using 3×50 µL ethyl acetate, evaporated to dryness once again and dissolved in 15 µL ethyl acetate for the purpose of analysis.

Analysis is carried out by injecting a 2 µL sample splitless onto a Rtx5-MS column (15 m×0.25 mm×0.25 µm, from Restek) with a 0.25 mm retention gap (methyl deactivation, from Analyt) and separated under the following operational conditions:

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Injector:	320 °C
Column temperature:	1 minute at 80 °C, then increase by 50 °C/min until 200 °C, 1 minute at 200 °C, increase by 30 °C/min until 260 °C, 1.5 Minuten at 260 °C, increase by 50 °C/min until 300 °C, 1.5 minutes at 300 °C.
Carrier gas:	Helium with a column pre-pressure of 90 kPa at 80 °C;
Interface temperature:	280 °C.

Detection is carried out by means of a mass spectrometer and negative chemical ionisation (reaction gas methane, approx. 1.4 Torr, ion source temperature 250 °C, electron energy 240 eV); selected ion monitoring (SIM).
Quantification of the amines is based on the deuterated internal standards. The following amines can be quantified in this manner:
N'-acetyl-4,4'-diaminophenylmethane, N'-acetylbenzidine, 2-aminofluorene, 3-amino fluoranthene, 1-aminopyrene, benzidine, 6-aminochrysene, 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, 3,3'-dimethoxyaniline, 3,3'-diaminophenylmethane, 4,4'-di aminophenylmethane, 3,3'-dichloro-4,4'-diaminodiphenylmethane, 2,4-toluene diamine, 2,6-toluenediamine.

Haemoglobin adducts of readily volatile aromatic amines (liquid/liquid) [22]

Haemoglobin (200 mg) from each test subject is weighed in a centrifuge tube and dissolved in 0.1 M sodium hydroxide (4 mL). A solution is prepared with hexane (20 µL) and the recovery standards 2 ng aniline-d_5, 2 ng 2-methylaniline-d_4, 0.5 ng 3-methylaniline-d_4, 1 ng 4-methylaniline-d_4, 1 ng 2,4-dimethylaniline-d_3, 1 ng 2,6-dimethylaniline-d_3, 1 ng [^{13}C]-4-chloroaniline, 1 ng 4-aminodiphenyl-d_9. After being shaken for one hour at room temperature, the aqueous phase is shaken with hexane (6 mL) for 3 minutes. In order to eliminate any possible intermediate phases the samples are centrifuged (5 minutes at 3000 g), frozen using liquid nitrogen and thawed. The hexane phase is drawn off and dried by passing it through a Pasteur pipette filled with sodium sulphate. The sodium sulphate is rinsed with hexane (1 mL). Pentafluoropropionic anhydride (PFPA) (5 µL) is added to the combined hexane extracts and after 10 minutes at room temperature (before evaporation the hexane-PFPA solution can be washed with 3 mL 1 M phosphate buffer pH 7.4), it is evaporated until it has just reached dryness in a stream of nitrogen. The residue is taken up in hexane (15 µL, with 1 ng PFPA-4-bromoaniline).
For the analysis a 2 µL sample is injected splitless onto a DB 1701 column (15 m long, inner diameter 0.25 mm, 1 µm film thickness, from J&W) with a methyl-silyl retention gap (1 m, inner diameter 0.25 mm, from Analyt), and eluted under the following conditions:

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Injector and interface temperature:	200 °C
Column temperature:	50 °C for 1 min, then increase by 50 °C/min to 200 °C, 1.2 min at 200 °C, then increase by 50 °C/min to 240 °C and 3.2 min at 240 °C.
Carrier gas:	Helium at a flow rate of 1.5 mL/min.

Detection is carried out by a mass spectrometer with negative chemical ionisation and methane as the reaction gas (approx. 1.2 Torr). The electron energy is 240 eV and the ion source temperature is 200 °C.

The following amines can be quantified with this method:
3-aminobiphenyl, 4-aminobiphenyl, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, 3,4-dichloroaniline, 3,5-dichloroaniline, 2,3-dimethylaniline, 2,4-dimethylaniline, 2,5-dimethylaniline, 2,6-dimethylaniline, 3,4-dimethylaniline, 3,5-dimethylaniline, 2-ethylaniline, 3-ethylaniline, 4-ethylaniline, 2-methylaniline, 3-methylaniline, 4-methylaniline, *α*-naphthylamine, *β*-naphthylamine, 4-(trifluormethyl)aniline, 2,4,5-trimethylaniline, 2,4,6-trimethylaniline.

References

[1] *Deutsche Forschungsgemeinschaft:* Analyses of Hazardous Substances in Biological Materials. Volume 1, Wiley-VCH Verlag, Weinheim (1985).

[2] *Deutsche Forschungsgemeinschaft:* Analyses of Hazardous Substances in Biological Materials. Volume 4, Wiley-VCH Verlag, Weinheim (1994).

[3] *Deutsche Forschungsgemeinschaft:* Analytische Methoden zur Prüfung gesundheitsschädlicher Arbeitsstoffe, Vol. 2 Deutsche Forschungsgemeinschaft, Looseleaf collection 13th issue, Wiley-VCH Verlag, Weinheim (1999).

[4] *Deutsche Forschungsgemeinschaft:* List of MAK and BAT Values, Report No. 35, Wiley-VCH, Verlag, Weinheim (1999).

[5] *E. Bailey, A. G. Brooks, I. Bird, P. B. Farmer* and *B. Street:* Monitoring exposure to 4,4'-methylenedianiline by the gas chromatography-mass spectrometry determination of adducts to hemoglobin. Anal. Biochem. *190*, 175–181 (1990).

[6] *M. S. Bryant, P. L. Skipper, S. R. Tannenbaum* and *M. Maclure:* Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. Cancer Research *47*, 602–608 (1987).

[7] *B. Falter, C. Kutzer* and *E. Richter:* Biomonitoring of hemoglobin adducts: aromatic amines and tobacco-specific nitrosamines, Ludwig-Maximillians-Universität München (1993).

[8] *C. Kutzer, B. Branner, W. Zwickenpflug* and *E. Richter:* Simultaneous solid-phase extraction and gas chromatographic mass spectrometric determination of hemoglobin adducts from tobacco-specific nitrosamines and aromatic amines, J. Chromatogr. Science *35*, 1–6 (1997).

[9] *M. Longo* and *A. Cavallaro:* Determination of aromatic amines at trace levels by derivatization with heptafluorobutyric anhydride and gas chromatography – electron-capture negative-ion chemical ionization mass spectrometry. J. Chromatogr. A *753*, 91–100 (1996).

[10] *G. Sabbioni* and *D. Schätze:* Hemoglobin binding of bicyclic aromatic amines. Chem. Res. Toxicol. *11*, 471–483 (1998).

[11] *W. Albrecht* and *H.-G. Neumann:* Biomonitoring of aniline and nitrobenzene. Hemoglobin binding in rats and analysis of adducts. Arch. Toxicol. *57*, 1–5 (1985).

PM3006730708

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[12] *G. Sabbioni:* Quantitative structure activity relationship of the N-oxidation of aromatic amines. Chem.-Biol. Interact. *81*, 91–117 (1992).
[13] *D. Schütze, O. Sepai, J. Lewalter, L. Miksche, D. Henschler* and *G. Sabbioni:* Biomonitoring of workers exposed to 4,4'-methylenedianiline or 4,4'-methylenediphenyl diisocyanate. Carcinogenesis *16*, 573–582 (1995).
[14] *D. Ringe, R.J. Turesky, P.L. Skipper* and *S.R. Tannenbaum:* Structure of the single stable hemoglobin adduct formed by 4-aminobiphenyl in vivo. Chem. Res. Toxicol. *1*, 22–24 (1988).
[15] *P.L. Skipper* and *S.R. Tannenbaum:* Protein adducts in the molecular dosimetry of chemical carcinogens. Carcinogenesis *11* (4), 507–518 (1990).
[16] *M. Törnqvist* and *H.H. Landin:* Hemoglobin adducts for in vivo dose monitoring and cancer risk estimation. J. Occup. Environ. Med. *37(9)*,1077–1085 (1995).
[17] *L. Ehrenberg, K.D. Hiesche, S. Osterman-Golkar* and *I. Wennberg:* Evaluation of the genetic risks of alkylating agents: tissue doses in the mouse from air contaminated with ethylene oxide. Mutat. Res. *24*, 83–103 (1974).
[18] *H.-G. Neumann:* Analysis of hemoglobin as a dose monitor for alkylating and arylating agents. Arch. Toxicol. *56*, 1–6 (1984).
[19] *G. Sabbioni* and *A. Beyerbach:* Haemoglobin adducts of aromatic amines and polyaromatic amines. J. Chromatogr. B. *744*, 377–387 (2000).
[20] *O. Sepai, D. Henschler* and *G. Sabbioni:* Albumin adducts, hemoglobin adducts and urinary metabolites in workers exposed to 4,4'-methylenediphenyl diisocyanate. Carcinogenesis *16*, 2583–2587 (1995).
[21] *O. Sepai, D. Henschler, S. Czech, P. Eckert* and *G. Sabbioni:* Exposure to toluene diamines from polyurethane-coated breast implants. Toxicol. Lett. *77*, 371–378 (1995).
[22] *G. Sabbioni* and *A. Beyerbach:* Determination of hemoglobin adducts of arylamines in humans. J. Chromatogr. B. *667*, 75–83 (1995).
[23] *Bundesärztekammer:* Qualitätssicherung der quantitativen Bestimmungen im Laboratorium. Neue Richtlinien der Bundesärztekammer. Dt. Ärztebl. *85*, A699–A712 (1988).
[24] *Bundesärztekammer:* Ergänzung der „Richtlinien der Bundesärztekammer zur Qualitätssicherung in medizinischen Laboratorien". Dt. Ärztebl. *91*, C159–C161 (1994).
[25] *G. Lehnert, J. Angerer* und *K.H. Schaller:* Statusbericht über die externe Qualitätssicherung arbeits- und umweltmedizinisch-toxikologischer Analysen in biologischen Materialien. Arbeitsmed. Sozialmed. Umweltmed. *33(1)*, 21–26 (1998).
[26] J. Angerer und *G. Lehnert:* Anforderungen an arbeitsmedizinisch-toxikologische Analysen – Stand der Technik. Dt. Ärztebl. *37*, C1753–C1760 (1997).

Authors: *J. Lewalter, W. Gries*
Examiners: *J. Angerer, G. Sabbioni*

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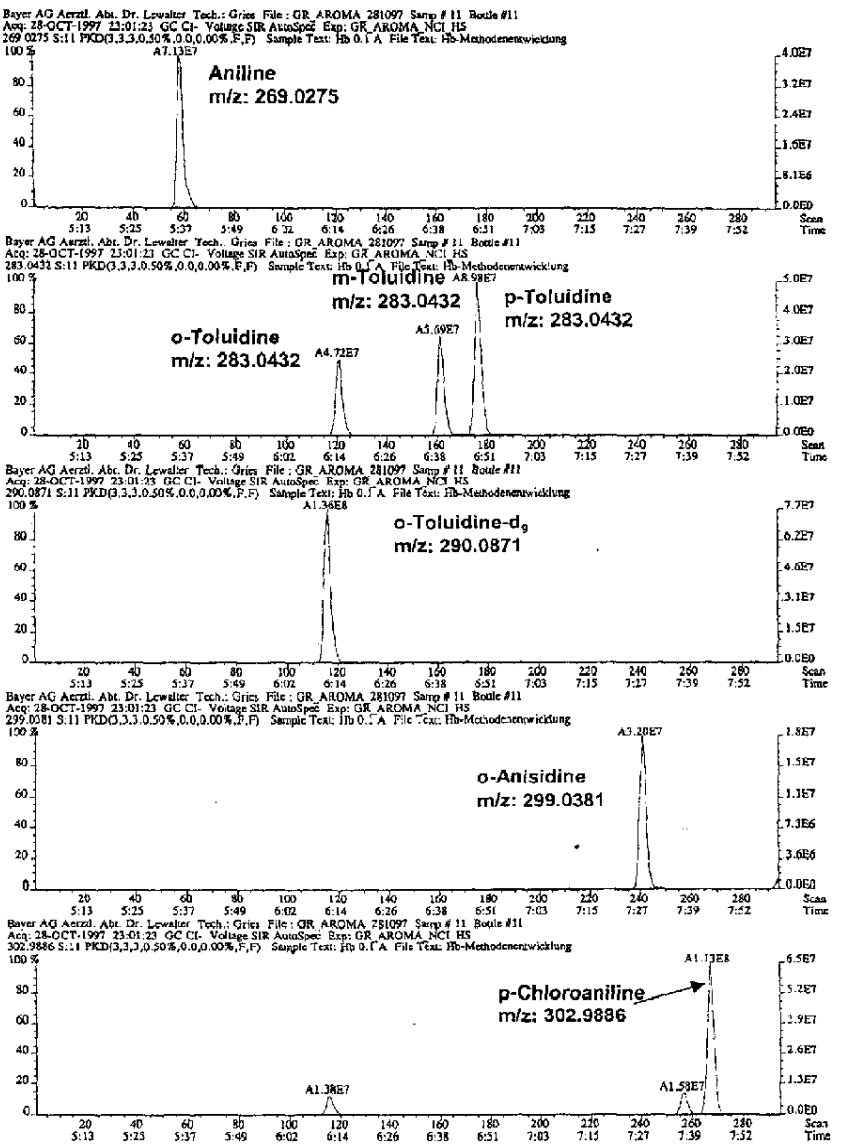

Fig. 1A. Example of a chromatogram (bovine haemoglobin spiked with 100 ng/L aromatic amines).

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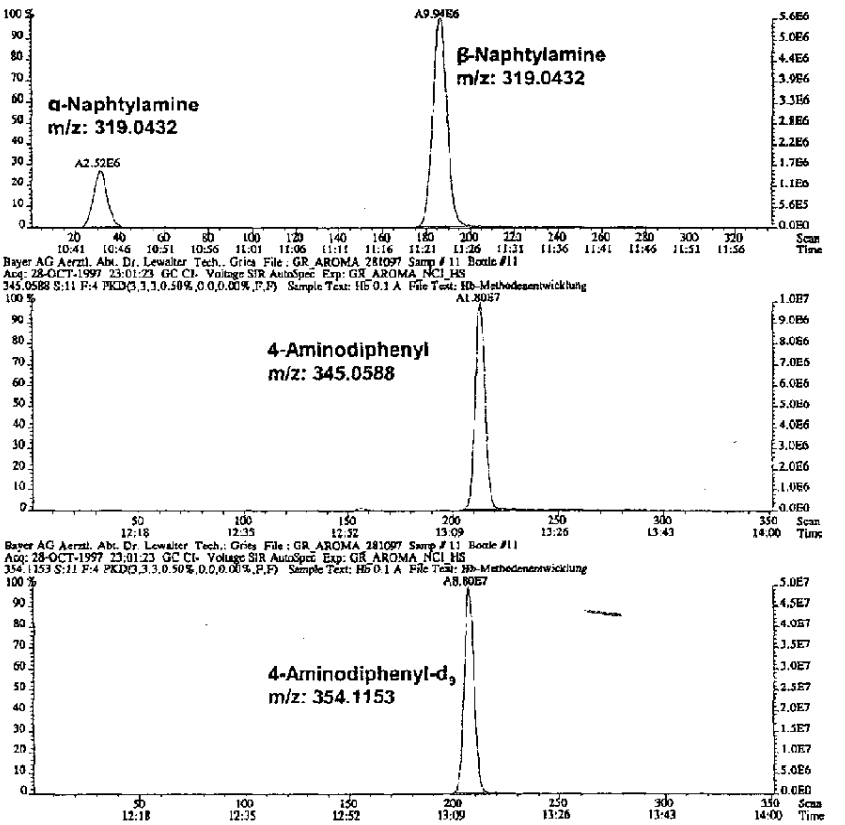

Fig. 1B. Example of a chromatogram (bovine haemoglobin spiked with 100 ng/L aromatic amines).

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Source: https://www.industrydocuments.ucsf.edu/docs/pyvj0001

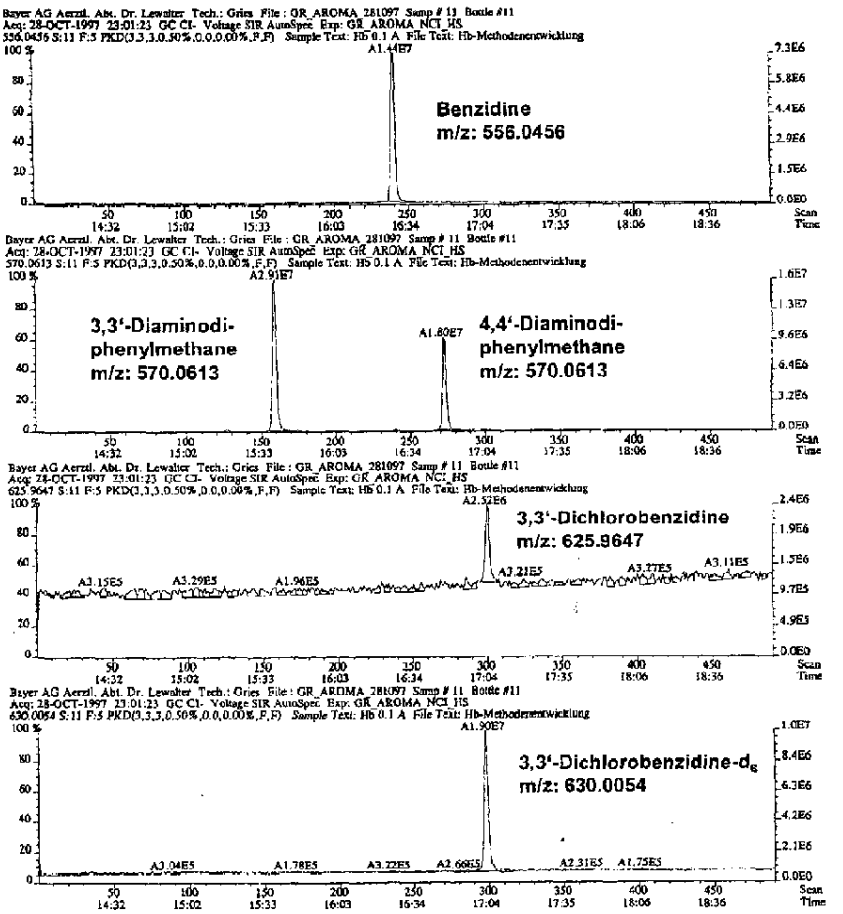

Fig. 1C. Example of a chromatogram (bovine haemoglobin spiked with 100 ng/L aromatic amines)

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